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Determination of Carbohydrate-Deficient Transferrin in Post-Mortem Blood Samples by Using Capillary Electrophoresis: Analytical and Interpretative Issues

S.S.D. MED/43 MEDICINA LEGALE

Coordinator: Dr. Federica Bortolotti
Tutor: Prof. Franco Tagliaro
Co-Tutor: Prof. Timothy M. Palmbach

Doctoral Student: Anthula Virginia Vandoros

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ABSTRACT

Post-mortem data on chronic alcohol abuse can be difficult to obtain due to putrefaction, inadequate background information at time of autopsy, and lack of distinctive pathological characteristics. However, diagnosis of chronic alcohol abuse can aid in death investigations and assist in establishing the cause and the manner of death. A well established biomarker of chronic alcohol abuse is Carbohydrate-Deficient Transferrin (CDT) (Bortolotti, 2006). Transferrin (Tf) is an iron transport serum glycoprotein consisting of two oligosaccharide chains. CDT is a subset of hypoglycosylated isoforms typically losing one or both glycan chains. Consuming 50-80 grams of alcohol a day for at least 7 days increases CDT serum values. After abstinence CDT returns to normal values in about 2-3 weeks. According to a sound body of literature, the diagnostic sensitivity of this marker ranges from 70-80%, while diagnostic specificity is about 100%. CDT value is expressed as percentage ratio of total transferrin; in non alcohol abusers being below 1.8% (Delanghe, 2009). Notwithstanding CDT is a worldwide recognized marker of chronic alcohol abuse, few studies are published on post-mortem applications largely because of the analytical problems caused by the post-mortem blood. The aim of this work was to investigate the possible use of this marker in post-mortem cases, developing an analytical method based on capillary zone electrophoresis suitable for the analysis of post-mortem samples.

498 post-mortem blood samples from the Office of the Chief Medical Examiner (OCME) in Connecticut were obtained with no restrictions. Measurement of CDT was done by capillary zone electrophoresis using a Beckman Coulter ProteomeLab PA 800 using a UV detector at 200 nm in the forward direction. All data was analyzed using 32 Karat software version 7.0.

Of the 498 samples, only 72 samples resulted in interpretable electropherograms. CDT value in these samples ranged from 0.43-13.04% (average 2.39%, SD 2.49%). 27 subjects showed CDT values higher than the established cut-off of 1.8% (used to distinguish between normal subjects and alcohol abusers) suggesting a condition of chronic alcohol abuse. Of the 30 cases with anatomo-pathological findings consistent with chronic alcohol abuse, 16 also had an increased %CDT. Moreover, 24 subjects indicated a recent alcohol intake as demonstrated by the determination of

blood alcohol content (BAC) (range 0.03-0.41%). Of these subjects, 18 were above the legal limit of 0.08%.

Capillary zone electrophoresis of post-mortem samples was useful in evaluating CDT to determine chronic alcohol abuse in autopsy cases.

INTRODUCTION

Consuming alcoholic beverages in a social setting has long been considered a way to relax and enjoy visiting with family and friends and has even been shown in some forms to help prevent cardiovascular disease in individuals. Alcohol, more specifically ethanol, has been a part of social gatherings for centuries helping to celebrate and commiserate. While ethanol itself is not an illegal substance it is understood that limits must be in place for certain activities while under the influence, above which the activity is illegal to perform (e.g. drive). These legal levels were created to reduce risky behaviors associated with increased amounts of inebriation leading to reduced cognitive ability and motor skills, lowered inhibition, and sleepiness among other characteristics.

Even though risks are associated with consuming high levels of ethanol, especially for long periods of time, many people still participate in the activity. As of 2009 in the United States of America (USA) according to the Centers for Disease Control and Prevention (CDC, 2009) 13.8 million adults abused alcohol, 8.1 million being alcoholics. Men are three times more likely to abuse alcohol than women and alcohol has been determined as the third leading preventable cause of death in the USA due to the approximate 79,000 deaths that occur every year.

As abuse of this readily available substance continues it is important to determine reliable ways to test individuals. Biochemical tests are performed routinely in drug screens due to the high usage of alcohol in society. Testing of individuals involved in high risk jobs or who are not permitted to consume alcohol while working is a valuable tool. Such jobs include ones utilizing heavy machinery, driving, aviation, and construction. Increases in biomarkers such as Gamma-glutamyl Transferase (GGT), Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), and mean corpuscular volume (MCV) signify liver damage which can be indicative but not limited to alcohol abuse. The markers are used widely in the clinical setting and are useful in first establishing a possible abuse pattern as the tests tend to be lower in cost and quick to complete. Monitoring and testing of living individuals has become routine but a void remains in the post-mortem area of chronic alcohol detection. Alcohol abuse is directly and indirectly associated with deaths (suicides, homicides, accidents) and can help medical examiners and medico-legal investigators

ascertain a decedent's behavior leading up to the incident. A negative blood alcohol concentration (BAC) at time of death does not necessarily mean that alcohol was not consumed on a regular basis before death, rather that it was not consumed close enough to the time of death and has had time to metabolize. Conversely, a positive BAC does not immediately deem the decedent a chronic alcohol abuser and this fact could be important to determine in an investigation. Moreover at time, there are often no conclusive pathological findings for chronic alcohol abuse as well as a lack of amnestic data for the medical examiner to pull data from. For these reasons a reliable method for CDT quantification in post-mortem samples would provide professionals a tool with which to determine chronic alcohol abuse ante-mortem.

ALCOHOL AND ALCOHOL ABUSE

Alcohol is consumed for a variety of reasons ranging from benign and extending to harmful excess. A typical drink is defined by any alcoholic beverage containing 14 g of ethanol (0.6 fl ounces), e.g. a 12 ounce beer (354 mLs), 5 ounces of wine (148 mLs), or 1.5 ounces of hard alcohol (44 mLs) (NIAAA, 2011). Alcohol usage comes in many forms such as abstainers (teetotalers), binge drinkers, moderate, and chronic. While abstainers of alcohol are clear, binge drinkers are defined as having 5 or more drinks in a period usually one hour or within two hours elevating their blood alcohol concentration above 0.8 (80 mg/dL). Acute intoxication of alcohol occurs when the consumption of alcohol exceeds the body's ability to metabolize and it continually circulates through the bloodstream and tissues until it is excreted. Chronic alcohol abuse typically results from the consumption of 50-80 g of ethanol daily for an extended period of time and can lead to far reaching physical and psychological damage.

Since alcohol is one of the most abused legal drugs it is important to understand its clinical classifications of abuse and dependence. The American Psychiatric Association's DSM-5, updated as of November 2013 (NIAAA, 2013), has combined the previously separate alcohol abuse and alcohol dependency (DSM-IV) into one disorder known as alcohol use disorder (AUD). AUD has three sub classifications entitled mild, moderate, and severe. Under this new system anyone who experienced two of the eleven criteria in a twelve month period are said to have AUD, the severity

further determined by the total criteria associated with the individual. The eleven criteria determined for evaluation of AUD are as follows:

- Alcohol is often taken in larger amounts or over a longer period than was intended.
- There is a persistent desire or unsuccessful efforts to cut down or control alcohol use.
- A great deal of time is spent in activities necessary to obtain alcohol, use alcohol, or recover from its effects.
- Craving, or a strong desire or urge to use alcohol.
- Recurrent alcohol use resulting in a failure to fulfill major role obligations at work, school, or home.
- Continued alcohol use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of alcohol.
- Important social, occupational, or recreational activities are given up or reduced because of alcohol use.
- Recurrent alcohol use in situations in which it is physically hazardous.
- Alcohol use is continued despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by alcohol.
- Tolerance, as defined by either of the following:
    - A need for markedly increased amounts of alcohol to achieve intoxication or desired effect
    - A markedly diminished effect with continued use of the same amount of alcohol
- Withdrawal, as manifested by either of the following:
    - The characteristic withdrawal syndrome for alcohol
    - Alcohol (or a closely related substance, such as a benzodiazepine) is taken to relieve or avoid withdrawal symptoms.

Alcohol dependence is clearly a more involved disease as the persons affected relinquish normal behaviors in the pursuit of their addiction. Alcohol abuse can be both acute and chronic; however, alcohol dependence results from habitual abuse of alcohol.

Determining alcohol abuse is of great interest in the forensic science field as it is often the precursor to many crimes and accidents. Alcohol plays important roles in many forensically relevant cases such as fatal motor vehicle accidents, workplace injuries, child placement, drowning, homicides, and suicides. Being able to understand and interpret both analytical results and psychological issues regarding alcohol abuse are integral to understanding actions and reasons associated with casework.

ALCOHOL METABOLISM

Ethanol is a small water soluble molecule that easily diffuses across all membranes and causes harmful effects in cells due to its ability to interfere with proteins and membrane structures. There are several metabolic pathways for the removal of alcohol from the body the main one being oxidative metabolism via hepatocytes (liver cells) with minor oxidative pathways utilizing catalase and cytochrome p450 as well as non-oxidative pathways such as those that form ethyl glucuronide (EtG), ethyl sulfate (EtS), phosphatidylethanol (PEth), and fatty acid ethyl esters (FAEEs). The majority of alcohol is metabolized in a two stage oxidative pathway (phase I) converting the alcohol to acetaldehyde via alcohol dehydrogenase then forming acetic acid by converting the acetaldehyde utilizing aldehyde dehydrogenase. A much smaller percentage of alcohol undergoes phase II glucuronidation or sulfate conjugation of ethanol. A percentage of the alcohol (~10%) is also excreted unchanged through the urine, sweat, and expired breath of the consumer.

First pass metabolism (FPM) is done predominately in the stomach with second pass metabolism (SPM) taking place in the liver, the majority of absorption occurring in the small intestine with alcohol being carried to the liver via veins from the stomach and bowels as well as the portal vein. Once the alcohol enters the liver the primary pathway of oxidative metabolism can start. The ethanol reacts with alcohol dehydrogenase (ALD) present in the cytosol and becomes acetaldehyde which is a toxic and highly reactive substance. In order to remove the acetaldehyde, aldehyde dehydrogenase (ALDH) reacts and forms acetate which leaves the liver and travels to the blood where it is further metabolized to carbon dioxide and water, then excreted (Zakhari, 2006).

Alcohol that is present in cells where there is no ALD or low levels of the enzyme such as brain and muscle cells use cytochrome p450 and catalase to eliminate the alcohol.

BIOLOGICAL MARKERS OF ETHANOL CONSUMPTION

Due to the far reaching health and societal effects of alcohol it is important to be able to measure alcohol levels in persons for various reasons. The many tests used to determine alcohol usage in persons are considered state markers while the enzyme content and genetic code for various proteins involved in alcohol metabolism are considered trait markers. Trait markers can indicate predispositions for alcoholism, higher levels of intoxication, or increased behavioral sensitivity to the consumption of alcohol (Jones, 2008). Some trait markers include mutations in alcohol dehydrogenase that increase the enzymatic rate of metabolism leading to more damage by acetalaldehyde due to rate limiting at the acetaldehyde dehydrogenase step. Levels of the neurotransmitter GABA present is another trait marker that is inherited, and studies have found that alcohol abusers have lower levels of than abstainers, indicating at least a tentative link with between GABA and chronic alcoholics (Peterson, 2004).

State markers depend on the consumption of alcohol, increasing with heavier use and decreasing with abstention. In forensic science, state markers test persons for acute and chronic intoxication in traffic violations, custody hearings, workplace fitness testing, motor vehicle homicides, suicides, and accidental deaths among other cases. State markers are used to determine fault, behavior before death or crime, fitness for placing a child, and monitoring levels for license reissuing (Bortolotti 2011). It can be important to make the distinction between an acute intoxication and a chronic abuse of alcohol, the latter more indicative of a person who will likely repeat their behavior. There are several state markers that can be used to test for acute and chronic alcohol abuse.

MARKERS OF ALCOHOL ABUSE

ALCOHOL & COGENERS

The most common chemical marker of acute intoxication is the direct measurement of ethyl alcohol (ethanol) itself in the blood and/or breath. Ethanol remains in the body for up to 24 hours in expired air, blood, and urine and indicates a recent consumption of alcohol but cannot decipher whether chronic or a binge event. Blood alcohol content (BAC) measurement of blood is the more reliable form of direct ethanol testing but can be more difficult since the blood must be taken by a professional (usually at a hospital) and not in the field. BAC is commonly determined by head space sampling gas chromatography utilizing a flame ionization detector (HS-GC).

A breath test can be performed in the field along with sobriety testing but has more variability depending on the respirations of the subject. This is a non invasive and easy test that allows for fast measurement of alcohol consumption. Most breath analyzers (breathalyzers) are designed using an average blood breath ratio (BBR) of 1:2100 for comparison of alcohol between the breath and blood partition.

Methanol is found in alcoholic beverages in concentrations of 0.1% (vodka) to 2% (brandies). An elevated level of methanol in blood (> 0.01 g/dL) can be indicative of chronic alcoholism (Jones 2008). It is also important to note that trace levels of ethanol and methanol are produced naturally in the body, especially during decomposition via bacterial action and are not necessarily indicative of alcohol consumption. An increased amount of alcohol use will lead to elevated levels of methanol in the blood and can be indicative of abuse.

METABOLITES OF ETHANOL

ACETALDEHYDE

Acetaldehyde is a direct metabolite of ethanol resulting from its reaction with alcohol dehydrogenase (ADH) and is quickly further metabolized to acetic acid by aldehyde dehydrogenase (ALD). After consumption of alcohol the levels of acetylaldehyde increase and can be measured. Genetic traits such as a mutant ALD2 gene found in asian populations can cause high levels of acetaldehyde to accumulate in blood as a result of poor binding to its substrate. The resulting levels of acetylaldehyde cause negative symptoms within the individual such as nausea, difficulty breathing,

tachycardia, and flushing (Jones, 2008). Those with the ALD2 gene mutation are intolerant to alcohol and medications that inhibit ALD2 have been used as aversion therapy to treat chronic alcoholics.

ETHYL GLUCURONIDE (EtG) & ETHYL SULFATE (EtS)

Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are direct metabolites formed after ethanol undergoes glucuronidation or sulfate conjugation following ethanol consumption and can be detected in blood, urine, and hair after consuming alcohol. EtG is formed by conjugating glucuronic acid to ethanol via catalysis by UDP-glucuronylsyltranferase while EtS is a conjugation between ethanol and sulfate catalyzed by sulfotransferase. Schmitt reported detection of EtG in serum up to 8 hrs after elimination of ethanol (Schmitt 1997). Halter reported that EtG and EtS can be detected in urine for days (three to eight times longer than ethanol) after ethanol consumption depending on initial dose (Halter 2008). Due to the shorter detection of EtG in serum and the longer detection of EtG and EtS in urine they present a way to determine both recent use of alcohol and an intermediate use bridging the short term marker of ethanol and the typical long term markers such as MCV, γGT, and CDT. In addition the short half life of EtG and EtS of 2-3 hours allows for these markers to be used for monitoring abstinence from alcohol use before and after surgery where alcohol use is prohibited. False positive of determination of EtG and EtS in urine have been attributed to ethanol containing mouthwashes and hand sanitizers among others.

Ethyl glucuronide has been determined as a marker for chronic alcohol abuse when measured in hair. The Society of Hair testing (SoHT) established guidelines for the determination of EtG in hair samples, citing a proposed cut-off value of 30 pg/mg to distinguish between use and abuse (Kintz, 2010). However the diagnostic specificity and sensitivity of hair EtG are scarcely known with variability in sweating patterns and hygiene affecting EtG concentrations (Tagliaro 2011). Bendroth published a study using EtG determination in hair at time of autopsy as a chronic abuse marker but concerns with the established cut-off made interpretation difficult as levels below do not necessarily indicate alcohol abstention (Bendroth 2008)

Berger et al recently published a report on using EtG as an indicator of long term ethanol consumption in hair and fingernails. Hair and fingernail samples were obtained from student abstainers, increasing risk drinkers, and high risk drinkers. Hair samples showed the best sensitivity among the high risk drinkers while fingernail testing showed a high sensitivity among both the increasing risk and high risk drinkers. While this study shows an interesting application of EtG it will be critical to improve specificity and sensitivity in certain areas (Berger 2014).

While EtG and EtS have both a high diagnostic specificity and sensitivity to ethanol, it is critical to note that there are false positives due to the ability to detect such low levels of the metabolites.

ALCOHOL CORRELATED ADDUCTS

ACETALDEHYDE PROTEIN ADDUCTS

Acetaldehyde protein adducts form when acetaldehyde generated during alcohol consumption react with proteins (hemoglobin, microsomal proteins) (Sillanaukee, 1992). While these protein adducts have been proposed as a marker for alcohol abuse, the lack of a validated method in serum has limited the investigation of these markers in the population.

FATTY ACID ETHYL ESTERS (FAEES)

Fatty acid ethyl esters (FAEEs) are formed by the esterification of free fatty acids with alcohol and are catalyzed by FAEE esterases. FAEEs are present in organs more prone to alcohol related damage such as the liver, pancreas, heart, and brain. It has long been known that FAEEs contribute to end organ damage after chronic consumption and interfere with normal working of mitochondrial membranes among other destructive effects (Laposata 1986).

FAEEs are formed after ingestion of alcohol and parallel the increase of ethanol in blood, however due to the fact that they accumulate in other tissues there can be secondary elimination pathways slower than ethanol. As a result FAEEs can be detected for up to 24 hours in acute intoxications and up to 100 hours after last alcohol intake in chronic alcohol abusers. The detection window of FAEEs can be useful in differentiating a binge drinker

(acute intoxication) and chronic alcohol abusers. FAEEs also accumulate in hair by deposition through sebum allowing for detection up to 2 months, yet it is important to note that like EtG certain hair products can increase levels of FAEEs in abstaining persons.
The Society of Hair Testing (SoFT) has established a set of cut-off values for the testing of FAEEs in hair samples using four esters (ethyl myristate, ethyl palmitate, ethyl oleate, and ethyl stearate). If the segment is 0-3 cm proximal to the scalp a value of 0.5 ng/mg is considered indicative of chronic excessive alcohol consumption; if 0-6 cm proximal to the scalp the cut-off value is 1.0 ng/mg (Kintz 2012). If the segment of hair is below 3 cm the results are to be interpreted with caution due to the issues of cosmetic treatments altering FAEEs.

PHOSPHATIDYLETHANOL

Phosphatidylethanol (PEth) is formed by transphosphytidylation of phosphatidylcholine in the presence of ethanol catalyzed by phospholipase D. Phosphatidylcholine is a protein found in cellular membranes and as such, PEth is incorporated into the membrane of red blood cells and can be detected after 2 weeks of alcohol consumption of around 50 grams or more per day. The half life of PEth in blood after abstention is approximately 4 days and it has a diagnostic sensitivity of greater than 90% (Hartmann 2006).
In 2001 Hansson performed a study measuring PEth levels in 85 autopsies, 70 of which tested positive (Hansson 2001). Detecting a direct metabolite of ethanol is extremely useful in the forensic context as it is a direct result of ante-mortem consumption; however, Aradottir observed in 2004 that postmortem formation of PEth occurred in the presence of ethanol. Samples need to be extracted within hours of autopsy and frozen at -80^{o}C to limit the reaction. Due to the implication of sample preparation limitations the useable window of PEth in postmortem cases must be watched closely.

SALSOLINOL

Salsolinol is synthesized from the reaction of dopamine and acetaldehyde by the salsolinol synthase enzyme or by its reaction with pyruvate. After

consumption of alcohol salsolinol levels increase in blood and have been proposed as a contributor to the addiction process of alcohol (Mravec, 2005). It is important to take not of the matrix tested as levels increase in blood, decrease in urine after acute alcohol consumption, and no significant difference in the brain of abusers and abstainers has been noted (Peterson, 2004).

PRODUCTS OF ALCOHOL-INDUCED METABOLIC CHANGES

5-HYDROXYTRYPTOPHOL (5-HTOL)

5-hydroxytryptophol (5-HTOL) is a metabolite of the neurotransmitter serotonin (via monoamine oxidase enzyme MAO), a cellular communication chemical. Alcohol and acetylaldehyde disrupt the metabolism of serotonin so that an increase of 5-HTOL occurs in people consuming alcohol versus abstaining and can be measured up to 24 hours after consumption. In 2004 Johnson et al used a ratio of 5-HTOL/5-HIAA (5-hydroxyindole-3-acetic acid) another metabolite of serotonin to determine ante-mortem ethanol consumption in post-mortem urine using LC-MS. The increase of serotonin metabolites in the urine indicated ethanol consumption prior to death (not attributed to post-mortem microbial formation) with levels above 15 pmol/nmol classifying as positive (Johnson 2004). Including 5-HIAA in the measurment of 5-HTOL is critical to reduce the false positives that can occur from dietary sources of serotonin (Helander 1995).

MEAN CORPUSCULAR VOLUME (MCV)

Mean corpuscular volume (MCV) is the measurement of the average size of erythrocytes (red blood cells). After sustained drinking of 4-8 weeks, the size of red blood cells increase; likely as a result of toxic effects on bone marrow and development of red blood cells (increased number of vacuoles) and decreased membrane fluidity (Ballard 1997). Alcohol can also contribute to vitamin deficiencies as it interferes with B12 and folic acid absorption. Due to the 120 cycle of most erythrocytes, MCV values take longer to return to normal after cessation of drinking alcohol.

γ-GLUTAMYL TRANSFERASE (GGT)

Gamma glutamyl Transferase (gGT) is a serum enzyme produced mainly in the liver and plays a significant role in the gamma-glutamyl cycle which is responsible for the synthesis and degradation of glutathione. It has been noticed that after exposure to increased alcohol intake (>60 g/day) for several weeks, the levels of gGT increase as a result of associated liver damage and release of gGT from the hepatocytes. After abstention, the levels return to normal range after 20-25 days (Rosman 1992). Whitfield noted in 2001 that there are several factors affecting gGT measurement such as hepatitis C, cirrhosis, carcinoma, cardiovascular disease, diabetes, obesity, and kidney failure. Whitfield also observed that there were some variations in levels of serum gGT among subject populations. Typically levels are higher in adult males than females, black subjects, smokers showed an average 24% increase, and women using oral contraceptives a 10% mean increase of levels (Whitfield 2001). While gGT itself is not highly sensitive or specific for the indication of chronic alcohol abuse, it is commonly used alone or in conjunction with MCV and AST/ALT for a first assessment of abuse due to the wide availability of testing methods in clinical and laboratory settings.

ASPARTATE AMINOTRANSFERASE (AST) & ALANINE AMINOTRANSFERASE (ALT)

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are enzymes involved in amino acid metabolism. These cellular enzymes are found in body tissues such as liver, heart, brain, skeletal muscle, and red blood cells. Clinically these levels are measured often and used as an indication of liver health, with a ratio of AST to ALT exceeding 2.0 suggesting liver damage. While AST and ALT are used as markers of chronic alcohol abuse, their increase results from an overall liver damage which can occur for a variety of reasons other than alcohol abuse, thus the diagnostic specificity and sensitivity are low.

CARBOHYDRATE DEFICIENT TRANSFERRIN (CDT): GENERAL ASPECTS

Human transferrin (hTf) is an iron-binding protein that circulates in the blood transporting iron throughout the system. HTf is mainly synthesized in hepatocytes and contains a polypeptide chain, two iron metal binding sites one at the c terminus and on at the n terminus, and two N-linked carbohydrate side chains. HTf is comprised of 679 amino acid residues with a molecular weight of approximately 79 kD with two c-terminal lobe N-glycosylation sites (asparginine-413 asparginine-611) responsible for binding two bi and/or triantennary carbohydrate side chains composed of N-acetylglucosamine, mannose, galactose, and sialic acid. The terminal sialic residues are the only charged molecules and give the various isoforms of hTf their names.

The largest isoform of circulating Tf is tetrasialo-Tf (75%) and contains two biatennary N-glycans with four sialic acid residues and a pI of 5.4. Minor glycoforms of hTf exist containing zero sialic residues (asialo-Tf, pI 5.9), one sialic acid residue (monosialo-Tf, pI 5.8), two sialic acid residues (disialo-Tf, pI 5.7), three sialic acid residues (trisialo-Tf, pI 5.6), and five sialic acid residues (pentasialo-Tf pI 5.2). Disialo-Tf, trisialo-Tf, and pentasialo-Tf are found in serum of normal subjects as well as traces of asialo-Tf, monosialo-Tf, and hexasialo-Tf.

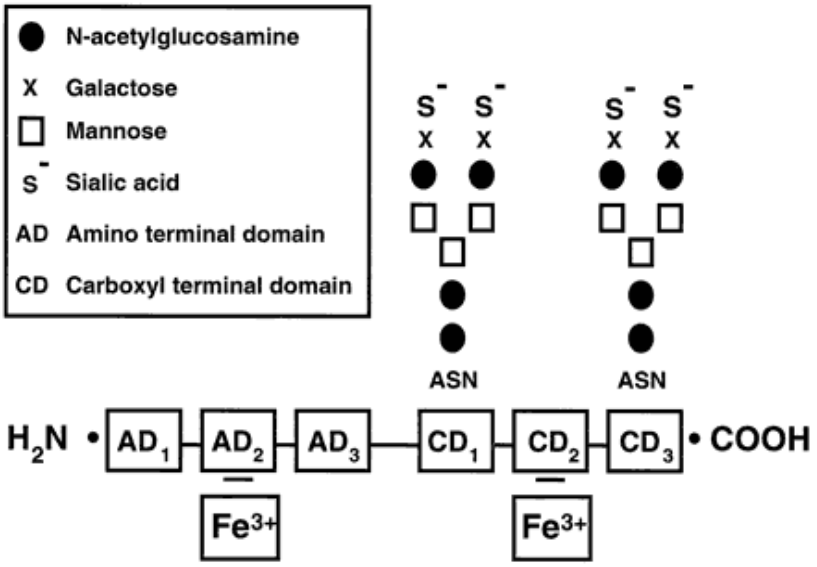

Figure 1. Human Tetrasialo Transferrin Structure

The metal ion binding sites fold into two structurally similar lobes, the N and C-lobes that contain conserved amino acids to aid in binding (two

tyrosines, one aspartic acid, and one histidine). Additionally two oxygen molecules given by a carbonate molecule stabilize the iron atom when bound to hTF. Iron binds to transferrin with high affinity at physiologically neutral pH (10^{-22} M) and has 3 possible forms, no iron bound (apotransferrin) and one or two iron molecules bound (holotransferrin), depending on the availability of Fe^{3+}. Once iron is loaded, transferrin binds to its cell surface receptors and is endocytosed where the pH inside the vesicle is lowered to ~5.6 releasing the iron (Byrne 2010). In a healthy individual approximately 30% of transferrin is saturated with iron, during iron deficiency more apo-Tf is present in the serum. Alternatively iron overloading (hemochromatosis) increases the amount of transferrin with Fe^{3+} bound in serum (De Jong, 1990).

The glycoforms of serum transferrin are characterized by their microheterogenicity, distinguished by isoelectric focusing (IEF) electrophoresis to discriminate based on their amino acid content, iron bound confirmations, and carbohydrate content of the branched chains. The N-glycan chains differ in their degree of branching carbohydrates, potentially binding 2, 3, or 4 structures terminating in a negatively charged sialic acid residues ranging from zero until 8. The approximate composition of each glycoform in normal serum is: asialo-Tf <1%, monosialo-Tf <1%, disialo-Tf 2%, trisialo-Tf 5%, tetrasialo-Tf 75%, pentasialo-Tf 15%, hexasialo-Tf 2%, heptasialo-Tf <1%, and octasialo-Tf <1%.

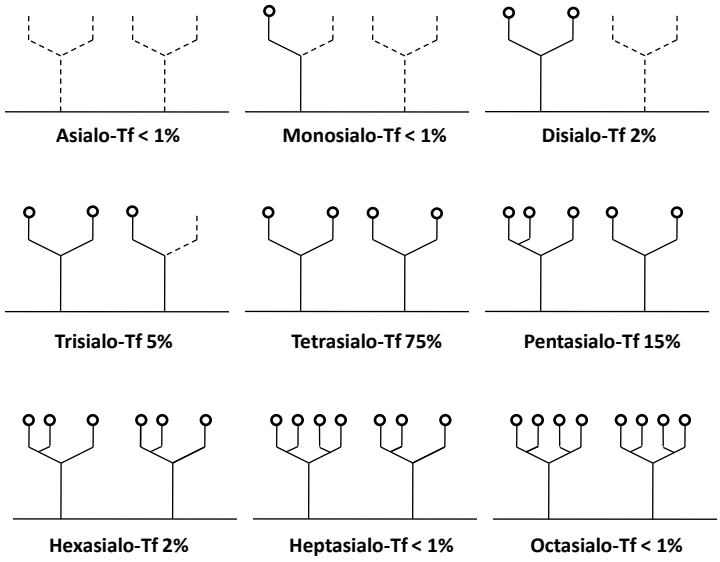

Figure 2. Microheterogenicity of Human Transferrin in Serum

Microheterogenicity of abnormal hTf glycoforms was reported by Stibler in 1978 in the cerebrospinal fluid of alcoholics, half of which also showed an increase in the disialo-Tf in serum (pI 5.7). The glycoforms that increased concentration after sustained alcohol consumption of >60 g/day were disialo-Tf, monosialo-Tf, and asialo-Tf. These lower glycosylated isoforms are called carbohydrate deficient transferrin and comprise less than 1.8% of total serum transferrin in a normal subject. In 2001 Bergen attributed loss of one or more glycan chains to the accumulation of CDT by using ESI-MS of Tf Isoforms after IEF, however Flahaut showed using MALDI-MS that both N-glycan chains (major) and sialic acid moieties (minor) were being lost after chronic alcohol abuse in disialo-Tf fractions (Flahaut, 2003).
Using current analytical techniques, IEF, capillary electrophoresis (CE), and high performance liquid chromatography (HPLC) asialo and monosialo-Tf fractions are not typically seen in healthy subjects. After chronic drinking of alcohol, the asialo-Tf can be noticeable along with a marked increase in disialo-Tf. Even knowing this, total transferrin is still calculated by adding the asialo, monosialo, and disialo-Tf values together.

Genetic variations in the transferrin molecules can occur as a result of amino acid substitutions in the polypetptide chain potentially affecting the

final charge of the molecule. At least 38 variants have been isolated and characterized based on their electrophoretic mobility, however only 4 of these show a prevalence of 1%. Of these main variant groups (B,C, and D) the C1 subgroup is the most common and gives no interferences with current analytical methods as its main isoform is of pI 5.4 (tetrasialo-Tf). Heterozygotes of transferrin have two peaks for each glycoform and can co-elute. This creates analytical problems especially with the BC and CD heterozygotes. The anodal B group can give a false negative result (lower pI) while the cathodal D group can give rise to false positives due to its amino acid substitution causing the pI to be >5.7 even though it possesses the same iron and carbohydrate content (tetrasialo-Tf) as the common Tf-C variants (Bean 1994). Plainly, subjects with Tf-BC variants will co-elute with non CDT isoforms of Tf-C variants even if heavy drinking is involved leading to false negatives and conversely subjects with Tf-CD variants will co-elute with CDT fractions of Tf-C variants even when no sustained alcohol consumption has occurred giving rise to false positives.

While it is clear in the literature that sustained alcohol consumption of >60 g/day for a period of at least 7-10 days will increase CDT, the biochemical mechanism for its increase is not well understood. Lakshman proposed that chronic exposure to alcohol affects cellular glycosylation, a critical and complex pathway in cells. Posttranslational modification of transferrin mainly occurs in the liver and requires several specific glycotransferases. Lakshman noted that the mRNA transcripts of sialytransferases in heavy drinkers were destabilized and reduced in number. As a result terminal sialic acid residues were not added to the branching carbohydrate chains as in normal subjects due to impairment of glycosylation leading to an increase of CDT (Lakshman, 1999).
Stibler and Borg showed that an increase in acetaldehyde concentration reduced the activity of galactosyl- and N-acetylglucosiminyl transferases involved in the beginning stages of N-glycan synthesis of hTf in the Golgi apparatus of liver cells (Stibler 1991). The increase of acetaldehyde in the oxidative metabolism of ethanol by ALD would lead to the accumulation of CDT due to impairment of glycosylation. Sillanaukee proposed after reviewing several studies that there is likely not one single event responsible for the increase in CDT levels, but likely a combination of protein transport and changes in enzyme activity (2001).

Ramskogler tested whether hepatocellular damage could be a reason for an increase of CDT levels by performing apoptosis experiments on 72 individuals suffering from alcohol toxic hepatic disorders. Along with CDT, gGT and liver enzymes (ASAT and ALAT) were measured as a marker for liver damage. While gGT and the liver enzyme levels increased and showed a strong correlation between them after acute hapotocyte damage, CDT did not suggesting that the increase of CDT is not directly linked with hepatocyte damage but hepatocyte metabolic impairment (Ramskogler 2004).

DIAGNOSTIC SENSITIVITY & SPECIFICITY OF CDT

In any analysis it is critical to know the diagnostic sensitivity (ability for the marker to be correctly identified) and the specificity (ability for the marker to not be identified when absent) of analyte being tested with regard to the specific pathology of interest. In the case of chronic alcohol abuse, the ideal marker with a high sensitivity and specificity will be able to determine chronic alcohol abusers from moderate and non-drinkers as well as from persons suffering from other conditions that could give a false positive (liver cirrhosis, Tf-variants, pregnancy), be a direct metabolite or be directly affected by ethanol increase, and be dependent on the amount of alcohol consumed.

While CDT is a good indicator of chronic alcohol abuse, there are other pathologies for which CDT levels can increase. Severe hepatic failure as a result of chronic viral hepatitis, primary biliary cirrhosis, and hepatocellular carcinoma can lead to false positive CDT results due to the overall low levels of transferrin present skewing the value of CDT. Also Congenital Disorder of Glycoyslation (CDG), hormone differences in woman (during pregnancy, menopause, or while on oral contraceptives), and alcohol intake in iron-depleted patients can lead to increased levels of CDT.

A phenomenon called disialotransferrin-trisialotransferrin-bridging, prevalent in patients with cirrhosis of the liver, is an incomplete separation between the disialo-Tf and trisialo-Tf peaks. Di-tri-bridging is seen using HPLC and capillary electrophoresis methods at a frequency of ~0.8%. This strange occurrence was linked to an alteration in the glycosylation of the carbohydrate moiety, adding fucose which has been shown to increase retention times (Landberg, 2012).

Bianchi reported that gestational trimester and %CDT is independently associated to gestational week using the HPLC candidate reference method. An increase in %CDT in abstainers is marked (0.52%) from first trimester until the third trimester (Bianchi, 2011). Kenan et. al showed that an increase of disialo-Tf in the third trimester of pregnancy combined with a decrease in trisialo and tetrasialo-Tf could lead to false positives in pregnant women (Kenan, 2011). Bakhireva reported that 35 subjects joining a study at an average gestational point of 7.3 weeks showed a mean increase of 0.18% from 1.49% CDT by the end of their term. Using the approved cutoff value for their method of 1.7% (CDT/Total Tf x 100), this put 45.7% of the subjects positive for CDT (Bakhireva, 2012). Due to the nature of CDT increase in pregnant women it is important to be careful when reporting values.

Bergström and Helander reported a statistically significant but small difference in hTf composition between genders using an HPLC method. Men showed slightly higher levels of tetrasialo-Tf as compared to women and slightly lower pentasialo-Tf than women however the amount of disialo-Tf to total transferrin did not differ significantly. They also noticed that those with a body mass index (BMI) above 30 showed a significantly higher mean value of disialo-Tf (Bergström, 2008).

Traditional makers for chronic alcohol abuse mean corpuscular volume (MCV), γ-glutamyltransferase (gGT), and aspartate aminotransferase & alanine aminotranferase (AST/ALT) are prone to sensitivity and specificity issues. In general gGT has a sensitivity of 34-85%; however it has a low specificity since levels increase for non alcoholic liver disease, obesity, diabetes mellitus, and several other pathologies. MCV has a high specificity (80-90%) when determining chronic alcohol abuse as it takes 30 days of >60g ethanol consumption to slowly increase levels but suffers from low sensitivity because certain populations (e.g. vitamin B12 deficiencies) will be positive for increased MCV but not necessarily a direct result of chronic alcohol abuse.

In comparison, CDT shows a high specificity for chronic alcohol abuse as it requires at least 7-10 days of >60g alcohol consumption before appearing in serum of healthy subjects and has a relatively long half life. When evaluated against traditional markers, CDT measurement was surpassed only by the combination of CDT with gGT in cases of differentiating “moderate” from “heavy” drinkers (Sillanaukee 2001).

CDT: PRE-ANALYTICAL PROBLEMS

Pathologies do play a role in CDT levels as well as differences in populations; however, it is important to note that pre-analytical handling can also lead to variations in CDT measurements. Several authors have written about the importance of storage temperature and time, freeze-thaw cycles, type of collection tubes (anticoagulant or not), and time from sample collection and centrifugation. In general most authors agree that CDT is stable at -20^{o}C for months after centrifugation of whole blood and serum is removed and days to a weeks at 4^{o}C; also freeze thawing does not appear to effect the samples greatly unless done multiple times. Stibler reported in 1986 that heparin impaired in vitro Fe^{3+} saturation and a 25% increase of CDT was found after 3 days at room temperature storage. Arndt reported in 2001 that samples centrifuged 1 hour, 24 hours, 48 hours, and 144 hours after collection showed a marked increase in CDT values (Arndt, 2001). This could have been due to bacterial activity of neuraminidase cleaving parts of the glycan chains.

CDT: ANALYTICAL METHODS

There are several analytical methods available to determine CDT in serum, the most frequent being immunoassay, capillary electrophoresis (CE), and high performance liquid chromatography (HPLC). In routine laboratory work it has been determined that immunoassay should be used only as a screening method later confirmed by CE and/or HPLC methods. While CE and HPLC have been show to have good correlation, the Working Group on Standardization of Carbohydrate-deficient Transferrin of the International Federation of Clinical Chemistry and Laboratory medicine (WG-CDT-IFCC) declared HPLC the candidate method based on its superior selectivity of detection (460nm λ-specific for Fe^{3+} bound Tf) vs. CE's 200-214nm λ at which all proteins absorb (ultraviolet UV).

IMMUNOMETRIC METHODS

Utilizing immunoassays to measure CDT is a common method, especially in clinical settings, due to their ease of use and suitability for routine analyses.

Stibler et al first analyzed CDT by performing isoelectric focusing (IEF) separating the isoforms based on their pIs, followed by immunoelectrophoresis, and finally western blot for visualization of proteins. A separation step via anion exchange micro column was required before IEF to isolate CDT (pI >5.7) from serum. Alden et al demonstrated that affinity chromatography (micro-column separation) of samples from the Axis-Shield %CDT immunoassay left a marked amount of trisialo-Tf in samples potentially creating false positive CDT values (Alden 2005).

In an effort to remedy the problem of poor separation, complex electrophoretic stages, and enhance robustness several kits were designed that are exploit ion-exchange microcolumns to remove non CDT isoforms followed by various immunochemical detection methods (enzyme, radioimmunoassay, turbidimetric). While increasing productivity, a main concern of most CDT immunoassays still remained; that the antibodies used recognized all transferrin glycoforms, not specifically CDT epitopes and were reliant on a good separation of CDT from total transferrin prior to detection. Helander reported on the fact that genetic variants were not distinguishable in immunoassay testing because CDT isoforms are not resolved and visualized as they are in CE or HPLC (Helander 2001). In addition, the selectivity of separation cartridge technology in kits in conjunction with various detection methods increases variability making it difficult to fully compare and standardize results, something crucial in forensic medicine when the legal system is involved.

This was improved by the creation of the N Latex CDT direct immuno-nephelometric assay developed by Siemens Healthcare Diagnostics (Marburg, Germany) which uses monoclonal antibodies and CDT-like antigens that will agglutinate if no CDT is present in the sample being tested (otherwise it will not) and can easily be read with a spectrophotometer. Further increasing its appeal there is no sample pretreatment and associated spectrophotometers can be used with bar-coded sample tubes automating the testing process.

CHROMATOGRAPHIC METHODS

Taking advantage of the fact that transferrin isoforms differ in charge based on their terminal sialic acid residues, chromatographic methods were developed to increase specificity. Separation of the less abundant CDT

isoforms from the higher total serum transferrin was critical to achieve higher specificity and selectivity as well as to determine Tf genetic variants. Jeppsson et al created a method based first on anion exchange separation using columns followed by salt gradient elution and direct detection of CDT at a wavelength of 460 nm (Tf-Fe^{3+} complex) (Jeppsson 1993). This method was improved on by Helander et al by modifying the salt gradient elution, changing the anion exchange column, and detecting CDT at 470 nm (Helander 2003). The high specificity of HPLC warranted it to be chosen as the candidate reference method for CDT testing by the Working Group on Standardization of Carbohydrate-deficient Transferrin of the International Federation of Clinical Chemistry and Laboratory medicine (WG-CDT-IFCC) as previously stated.

ELECTROPHORETIC METHODS

At the discovery of carbohydrate-deficient transferrin, the method of separation was isoelectric focusing which precisely separated Tf isoforms based on their pI values. While very specific and still considered a gold standard separation method today, there were inherent issues in the detection and quantitation steps following IEF not to mention the time of analysis involved. Western blot analysis and/or staining of gels with silver (or other) stains were only as sensitive as the reagent and detection method. Also, the quantitative aspect of these off line detection techniques is not as precise or reproducible as liquid chromatography or capillary electrophoresis techniques, and therefore not commonly used in routine analysis.

Many methods involving capillary electrophoresis are being used to determine CDT values, especially capillary zone electrophoresis (CZE), separating the various isoforms based on their electrophoretic mobilities (charge/mass). In 1998 Tagliaro et al proposed a method for the separation and detection of CDT by CZE using an uncoated capillary and 100 mM sodium tetraborate buffer at pH 8.3. Samples were incubated with $FeCl_2$ and sodium bicarbonate to saturate the Tf iron binding sites (to decrease variations in mass between molecules) basing the separation on overall charge contributed by the terminal sialic acid residues. A direct on line detection of Tf was done using a UV detector at 200 nm wavelength (Tagliaro 1998). In 2000 a further improvement to this method was done

adding diaminobutane (DAB) to the buffer, reducing protein interaction with the capillary walls (silanol groups) increasing analytical sensitivity and selectivity (Crivellente 2000).
Several commercial kits have become available for the testing of CDT using capillary electrophoresis attempting to simplify sample prep and analysis time in order to increase routine use. One such kit, CEoFix CDT (Analis Namur, Belgium) released in 2001, employed a dynamic double coating of the fused silica capillary (i.d. 50 um, 60.2 cm), buffer system (pH 8.4) provided by the manufacturer containing ferric iron removing the pre-analytical step required for saturation, and took approximately 8 minutes of analysis time. Tagliaro et al later critiqued that even though the reproducibility of the kit was high, there was not a good separation between disialo-Tf and trisialo-Tf especially when trisialo-Tf was in higher concentrations suggesting that the decrease in resolution was not worth the advantage of direct measurement of serum (Tagliaro 2002). Shortly after Legros published a slightly modified version of the manufacturer's protocol including an iron saturation step prior to sample analysis allowing for good separation of transferrin isoforms (Legros 2003).
Many experiments testing various dynamic coatings (diaminobutane, spermine, CEofix® double coating, diethylenetriamine) and buffers have been published in the literature attempting to improve analytical sensitivity and reproducibility whilst maintaining relatively short analysis time. In fact after Lanz pointed out areas where the Analis CEofix® kit could be enhanced, an improved version was released in 2003 that obtains better separation between disialo and trisialo-Tf. A huge advance in the routine analysis of CDT came in the form of the Capillarys multicapillary electropherograph created by Sebia (Evry, France). A fully automated analysis and quantification can be performed including an iron pretreatment and direct UV detection at 200 nm simultaneously in seven channels.

MASS-SPECTROMETRIC METHODS

While mass-spectrometric analysis of CDT Mass-spectrometric analysis of human transferrin has been used to determine the molecular structure of the released carbohydrate chains, particularly in the case of disorders associated with abnormal Tf (carbohydrate deficient glycoprotein

syndrome, CDGS), it has never really been adopted due to its complexity and difficulties with quantitation. In 2003 Kleinert et al isolated transferrin using ion-exchange HPLC with UV detection followed by concentration of the transferrin fraction and detection via liquid chromatography-electrospray mass spectrometry. They were able to measure up to ten isoforms and apply the method to samples determining persons with congenital disorders of glycosylation (CDG-Ia, Ib, Ic, Ie, If, and IIa) as well as other pathologies (Kleinert 2003).

In 2005 Alden et al used matrix assisted laser desorption ionization time-of flight (MALDI-TOF) to determine the composition of eluate from the Axis Shield immunoassay (Axis Shield, Oslo Norway). According to the manufacturer the assay only measures a-, mono-, and disialo-Tf after separation via ion-exchange cartridges and turbidimetric detection (%CDT determined), however Alden measured trisialo-Tf in the eluate, potentially contributing to false positives (Alden, 2005).

Castillo Busto published an article using HPLC with online coupling of an inductively coupled plasma mass spectrometer (ICPMS) that allowed for the screening of transferrin molecules by atomic presence of iron only allowing them to be further characterized (lower detection limit of 0.03 μMTf) via MALDI-TOF and electrospray mass spectrometry (ESI-Q-TOF). MALDI-TOF proved to be sensitive to analyte concentration but ESI-Q-TOF gave a unique profile of CDT to apply towards diagnosis of alcoholism (Castillo Busto 2005). As previously mentioned, Landberg et al utilized MALDI-TOF to determine the composition of CDT samples containing di-trisialo-Tf bridging in 2012 and discovered a fucosylation of the carbohydrate branches (Landberg 2012).

FORENSIC APPLICATIONS OF CDT TESTING

A major concern of legal medicine is the chronic abuse of alcohol involving both living and dead persons. Testing a person for chronic alcohol abuse is important in cases involving license re-issuing, child custody hearings, workplace fitness (as in testing a pilot), and workplace safety (operating heavy machinery) among several others. Bortolotti et al. tested the reliability of CDT as a marker for risk of driving under the influence in 2007. Their study showed that in the control group 96.1% of subjects were negative for CDT levels above the cutoff while only 40% were negative in

the group of subjects stopped for drunken driving (Bortolotti 2007). This study, as well as others that are similar, support the measurement of increased CDT levels as a risk of drunk driving even though not objective in their findings. In 2014 Maenhout et al performed a study called Recidivism of Alcohol Impaired Driving or ROAD following 517 randomly selected drivers in Belgium who previously had their licenses confiscated. The study followed the drivers for 3 years and not only % CDT values were measured but also gGT, AST/ALT, and MCV. After performing logarithmic regression on their samples and using 1.6% CDT as their upper limit of normal (ULN), they determined that CDT was useful to assist in the prediction of recidivism for drunken driving. When gGT and ALT values were also determined after admittance into license re-granting programs, they were found useful for predicting recidivism but not to the extent of CDT (Maenhout 2014).

CDT determination is also useful to test persons involved in a job that requires strict safety measures such as a pilot or public transportation driver. Fustinoni et al showed a high specificity and sensitivity of CDT for predicting alcohol abuse among drivers but stated that a high CDT level is not a definite indicator of abuse (Fustinoni 2009). However it is clear that being aware of an employee's habits is useful when deciding if they can continue a job where others' and their own safety are at risk.

POST-MORTEM APPLICATIONS OF CDT TESTING

Death is directly or indirectly a result of alcohol abuse in many forensic cases especially homicides, suicides, and accidental deaths (including traffic accidents) and can give information to investigators. Half of all alcoholics are estimated to have a negative BAC at time of death (Sadler 1996). While a useful tool to determine ante-mortem habits, testing for alcohol abuse can prove difficult due to the short time frame of ethanol in the system, inadequate background information, and often lack of anatomo-pathological findings during autopsy. Commonly associated pathological findings in the liver (fatty liver, cirrhosis), pancreas (atrophy), brain (atrophy), and heart (hypertrophy, fibrosis) are not only indicative of alcohol abuse but have many underlying pathologies that could contribute.

Major issues in the testing of post-mortem samples, especially blood, is that they are susceptible to bacterial action due to decomposition,

autolysis, and redistribution of analytes due to free diffusion across membranes after loss of membrane potential. It is critical to know when given an analyte concentration whether it could be a result of post-mortem distribution. Under-estimated values can occur when the analyte in question has been diluted after release from where it was concentrated (for example in an organ) or overestimated because it has migrated and concentrated to an area of collection falsely increasing the result. The act of decomposition itself creates alcohols and can interfere with testing for ethanol post-mortem. Ethanol can also be produced by bacteria in a stored sample after collection especially when not stored or preserved properly (Gill, 2005).

In the case of post-mortem blood samples they are not like traditional whole blood in that the cells within have begun to rupture (autolyse) and release their contents. Of interest, the red blood cells which make up approximately 45% of whole blood burst releasing hemoglobin (an iron carrying oxygen transport protein). This causes the normal separation of serum from coagulation factors and cellular components to be impaired; not obtaining a clear separation as would be done ante-mortem which could pose problems for the forensic analyst.

To address some of the questions plaguing post-mortem blood samples with respect to CDT testing Simonnet et al tested several variables including effect of hemolysis, site collection, and sample storage. Addition of hemoglobin to samples was not shown to effect the CDT results however hemolysis of the samples showed a decrease of CDT vs. the non-hemolysed sera likely due to the dilution of red blood cells' contents which does not contain transferrin. As for site of collection, no significant difference was found between cardiac or femoral blood CDT levels. Storage of CDT for two weeks at +4^{o}C or -20^{o}C was shown to have no significant effect on the CDT values, however repeated freezing and thawing cycles showed a decrease in the value of CDT and should be avoided if possible (Simonnet 1999).

In order to further investigate the stability and post-mortem distribution of CDT, Rainio et al performed a study on 280 serum samples from 70 cadavers testing different retrieval sites (femoral blood prior to autopsy, right femoral blood at autopsy, left femoral blood at autopsy, and vena cava) and compared capillary zone electrophoresis (CZE) and HPLC methods. Their findings showed that CDT was stable up to several days of cold storage and the results were not significantly different amongst

collection sites, reconfirming that post-redistribution was not a factor on the analysis. Previously CDT was shown to remain stable up to 96 hours in post-mortem samples and Rainio corroborated this data with an average post-mortem interval (PMI) of 95 hours (range 39.2-152.4) (Rainio 2008). CDT has also been measured in vitreous humor by Berkowicz et al and found to be a good marker for chronic alcohol abuse. The fact that the vitreous humor resides in its own compartment segregated from bacterial action is helpful, however the shorter window for collection of sample limits it use (Berkowicz 2000).

EXPERIMENTAL PROCEDURES

INTRODUCTION AND AIM

Determining carbohydrate deficient transferrin at time of death can aid investigations, allowing for a more detailed view of ante-mortem behaviors. At this time, little testing has been done on post-mortem blood determination of CDT. Capillary zone electrophoresis offers a relatively quick and robust method with which to garner this information.
A study of post-mortem samples obtained from the medical examiner and separated by CZE was performed to determine the usefulness of CDT as a post-mortem indicator of ante-mortem chronic alcohol abuse.

MATERIALS & METHODS

CHEMICALS

All reagents used for buffer preparation were from Sigma Aldrich (St Louis, USA). A stock solution of 564 mM boric acid brought to pH 8.15 by 6 M NaOH was prepared weekly and kept at room temperature for use. A 90 mM solution of 1-4-diaminobutane (DAB) was prepared in 0.05 M HCl and stored at room temperature. In order to obtain iron saturation serum was pretreated with a ferric iron solution contained in the CEofix™ CDT kit (Analis, Namur Belgium).
For acid treatment of samples, stock solutions of 0.2 M HCl and 0.1 M sodium tetraborate were made and kept at room temperature. Samples were centrifuged in Amicon Ultra centrifugal filter units with a 0.5 mL capacity and 50 K molecular weight cutoff (Millipore,) using a microcentrifuge.

SAMPLE SELECTION AND PREPARATION

Samples were obtained with no restrictions from the Office of the Chief Medical Examiner (Farmington, CT). Samples were taken at time of autopsy and put in glass blood collection tubes (red top) and serum separator gel tubes (yellow top) made by BD vacutainer (Plymouth UK) and stored at 4^{o}C until pickup. Once samples were retrieved they were brought to the

University of New Haven (West Haven, CT) and were centrifuged for 10 minutes at 12000 x g. After centrifugation the "serum" fraction was pipetted off and put into 1.5 mL microcentrifuge tubes and stored at -20^{o}C until analysis. 10 µL of serum was pretreated with ferric iron at a dilution (1:8 for traditional method, 1:3 for acidic treatment method) and incubated at 4^{o}C for 10 minutes, after which it was centrifuged again to further clarify the sample. Samples were transferred to vials appropriate to analysis on the CE instrumentation.

Some testing of acid treatment of serum samples to remove interferences in the beta region of electropherograms where transferrin migrates was done. The main issue in this area is the interference of hemoglobin as it has a similar migration pattern to that of transferrin and is found in great abundance in hemolyzed post-mortem blood. In a paper published by Griffith they characterized the structure of hemoglobin proteins using ESI-MS. In the article they treated their samples with HCl to disrupt the heterodimers and dimers so that they could analyze the fractions (Griffith 2003). Adopting this, acid treatment of samples was done to breakdown the larger tetramers in an effort to alter their mobilities to a different region of the electropherogram. As such 0.2 M HCl was added to 300 µLs of serum until a pH of 3.5-4.0 was reached. After incubation overnight at 4^{o}C, samples were applied to 50 k molecular weight cutoff filter units and spun at highest speed in a microcentrifuge for 15 minutes. Post centrifugation samples were brought back to a physiological pH (7.5-8.5) using 0.1 M sodium tetraborate and incubated overnight at 4^{o}C. Bringing the pH back to physiological levels was done to regain transferrin conformation.

ELECTROPHORETIC CONDITION

Separation was achieved using a Beckman Coulter P/ACE-MDQ capillary electropherograph (Beckman Coulter, Fullerton CA) operating a UV-Visible detector at wavelength of 200 nm. Instrumentation control, data acquisition, and analysis were all done using 32 Karat software provided by the manufacturer (Beckman Coulter). Polyamide coated fused silica capillaries were obtained from Composite Metal Services Ltd. (West Yorkshire, UK).

The conditions for separation are as follows. A 60 cm polyamide coated fused silica capillary with an internal bore of 30 µm and effective length of

50 cm; running buffer composed of 154 mM boric acid containing 6 mM DAB at pH 8.15; capillary temperature of 25^{o}C; detection by UV-Visible at 200 nm; injection with positive pressure at 0.5 psi for 30 sec; voltage +30 kV; normal polarity.

At the start of each day the capillary was conditioned as follows to increase reproducibility and capillary regeneration: 20 min 1 M NaOH, 20 min H_2O, 20 min 564 mM boric acid pH 8.15, 20 min running buffer pH 8.15. Before each sample run a shorter rinse program was used with the same components at reduced times (3 min) to maintain reproducibility.

Valley to valley integration of peaks measuring the transferrin glycoforms present ranging from asialo-pentasialo-Tf was performed to obtain %CDT. %CDT was calculated by measuring and adding the areas under the curve of asialo-, monosialo-, and disialo-Tf and dividing it by the total area of the Tf glycoforms (a-,mono-,di-, tri-, tetra-, penta-), finally multiplying by 100 to get percentage. A cutoff value based on literature using serum and %CDT, to discern between normal or elevated was determined to be 1.8% (Delanghe 2009). All statistical analyses were performed in Excel with TTEST parameters being unpaired, one-tailed, unequal variance p values <0.05 considered statistically significant.

RESULTS AND DISCUSSION

GENERAL DATA COLLECTION

Separation by capillary zone electrophoresis to determine %CDT was done on post-mortem blood samples obtained at autopsy from the Office of the Chief Medical Examiner (OCME). 498 autopsies yielded a total of 996 samples (one red top tube, one yellow top) were tested, 89 obtained interpretable electropherograms, 12 of which were discarded since they could not be reproduced, 5 discarded due to their lack of autopsy information. 72 samples (14.5%) were further investigated to determine the significance of CDT testing as a forensic tool. No significant differences between the types of collection tubes were found when compared 71 times; the average difference between the two tubes was 0.06% ± 0.34%.

The average %CDT found in the 72 samples that were interpretable and could be repeated was found to be 2.39% ± 2.49% with a range of 0.43-13.04%. 16 samples were below 1% CDT and 27 samples measured above

the cutoff value of 1.8%, 13 of the 27 were above a level of 4%. In 24 samples a positive blood alcohol content (BAC), an indication of acute ethanol consumption (above 0.08% considered illegal for driving) at time of death was found with an average of 0.17% ± 0.11%, 18 of these subjects also had a %CDT above normal range. In 6 cases %CDT was not above the cutoff value, 4 of the 6 at 1.00% or below. No BAC testing was done in 5 cases, typical of the natural manner in which they died.

General demographic data of the samples showed a population composed of 16 females and 56 males with an average age of 41.57 years ± 17.5. No significance of gender on increased %CDT was found (p value 0.18). The racial profile of the population consisted of 54 Caucasians, 14 Black, and 4 Hispanic/White subjects and showed no statistical significance between Black persons or Caucasians for prevalence of increased %CDT (p value 0.18). BMI was calculated for subjects based on their height and weight and placed into categories based on the National Institute of Health (NIH) guidelines; below 18.5 underweight, 18.5-24.9 normal, 25-29.9 overweight, >30 obese. The average BMI was 26.99 ± 5.55 with a distribution of: 1 underweight, 26 normal weight, 26 overweight, and 19 obese subjects with increase of BMI in relation to an increase in %CDT not having statistical significance (p value 0.12).

Anatomo-pathological findings as reported by the medical examiners were reviewed when supplied. 30 cases showing signs of pathologies associated with chronic alcohol abuse (cirrhosis, fatty liver, cardiovascular disease, cardio hypertrophy) were found, 16 of them also had a %CDT above normal value showing a strong correlation between increased CDT levels with pathological findings indicative of chronic alcohol abuse (p value 0.01).

Manner of death was distributed as 37 accidental, 7 homicides, 20 natural, and 8 suicides.

Manner	Total #	%CDT AVE	%CDT STDEV	# Positive	%BAC AVE	%BAC STDEV	# Positive
Accident	37 (51%)	2.6	2.7	16	0.10	0.12	19
Homicide	7 (10%)	1.4	0.6	1	0.01	0.04	1
Natural	20 (28%)	2.9	2.8	9	0.01	0.04	3
Suicide	8 (11%)	1.2	0.5	1	0.01	0.04	1

Table 1. Distribution of Data Based on Manner of Death Including %CDT and BAC Values

A prevalence of increased %CDT values was seen in the accidental and natural cases whilst the smallest portion in homicide and suicide cases. It is difficult to draw conclusions based on these results because percentages of cases being homicide and suicide are low with respect to accident and natural making statements of linked %CDT increase unwise. It is interesting to note however that in 19 of the cases where an acute intoxication was reported, the manner of death is accidental with 13 having a corresponding increase in %CDT above the normal range. There is a high correlation of increased %CDT values and increased BAC values in the accidental death population (p value $1.2e^{-6}$). Accidental deaths resulted from overdose/intoxication in 17 cases, asphyxiation in 1 case, and multiple blunt traumatic injuries in 19 cases. Of those who suffered blunt force traumatic injuries, 10 were involved in motor vehicle accidents, 3 of those having a %CDT above the cut-off value.

Post-mortem interval (PMI) from the time pronounced dead until centrifugation are as follow:

	PMI AVE (hrs)	PMI STDEV	#	%CDT AVE	%CDT STDEV	# Positive Within PMI	% Positive Total
0-48	33.5	12.4	22	2.8	2.8	10 (45%)	37%
48.1-96	63.6	10.9	20	2.4	2.5	7 (58%)	26%
96.1-144	123.2	12.0	16	1.9	1.5	6 (37%)	22%
144.1-192	176.3	9.8	6	3.5	4.4	3 (50%)	11%
192.1>	267.3	55.9	5	1.8	1.8	1 (20%)	4%

Table 1. Post-Mortem Interval of Interpretable Samples with %CDT Values for Associated Time Ranges

As is shown by this table, a significant increase of %CDT values as PMI increases is not apparent as the CDT positive values are represented in fairly equal amounts, in fact most obtained at 144 hrs or below perhaps indicating some samples can still be measured beyond previously tested limits. Higher PMIs were a combinative effect of longer autopsy intervals and longer times held at 4^{o}C for pickup at the OCME. It would be wiser to have an in-house area to at least centrifuge the sample for immediate storage. While little difference amongst PMIs is a positive finding, the small sample size can by no means conclude that PMI has less of an effect on

%CDT than thought. It is interesting to note that interpretable data decreases as PMI increases as seen by total number of samples dramatically lowering after 144 hrs. The issue may not only be the affect of post-mortem interval on %CDT so much as the inability for the CZE method to separate and detect the CDT. As previously stated post-mortem serum samples suffer from hemolysis and can interfere with the detection of CDT. There are many proteins in whole blood that may not partition according to normal centrifugation due to the poor state of post-mortem blood, therefore these can also interfere with detection as a UV-visible detector measures proteins indiscriminately.

CASE EXAMPLES

Daily control runs consisting of a pooled transferrin sample with increased levels of CDT were run as a way to measure reproducibility since an internal standard could not be used. Intraday testing of a serum sample control (5 times) showed RSD values below 10% with interday testing of serum sample control RSD value below 20% for CDT% and absolute migrations times below 1% intra-day.

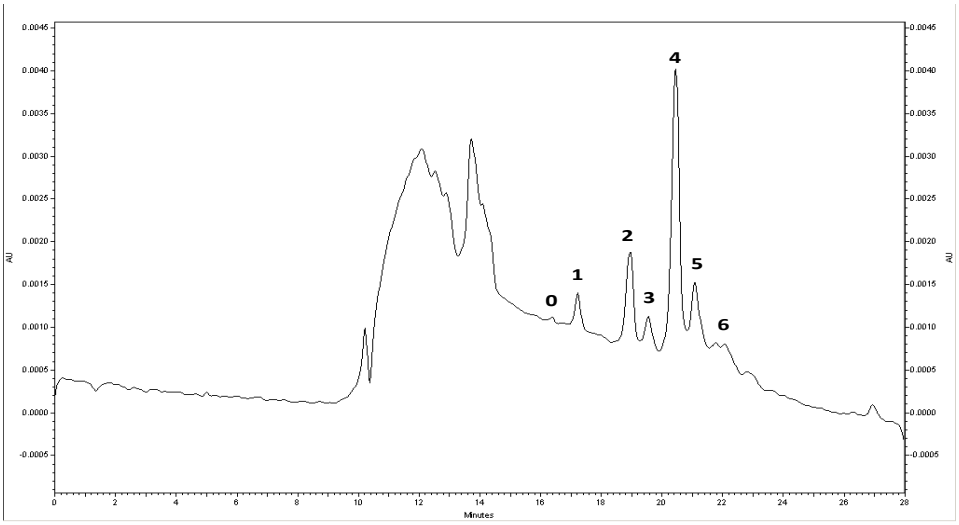

Figure 5. Electropherogram of Pooled Serum Control

Figure 5 shows an electropherogram of the daily control, transferrin glycoforms are represented as numbers 0=asialo-Tf all the way to 6= hexasialo-Tf. The control shows the resolution of asialo-Tf through trisialo-Tf with some peak overlap between tetrasialo and pentasialo-Tf. For this

reason tetrasialo and pentasialo are measured as one valley-to-valley integration event when calculating %CDT for this method.

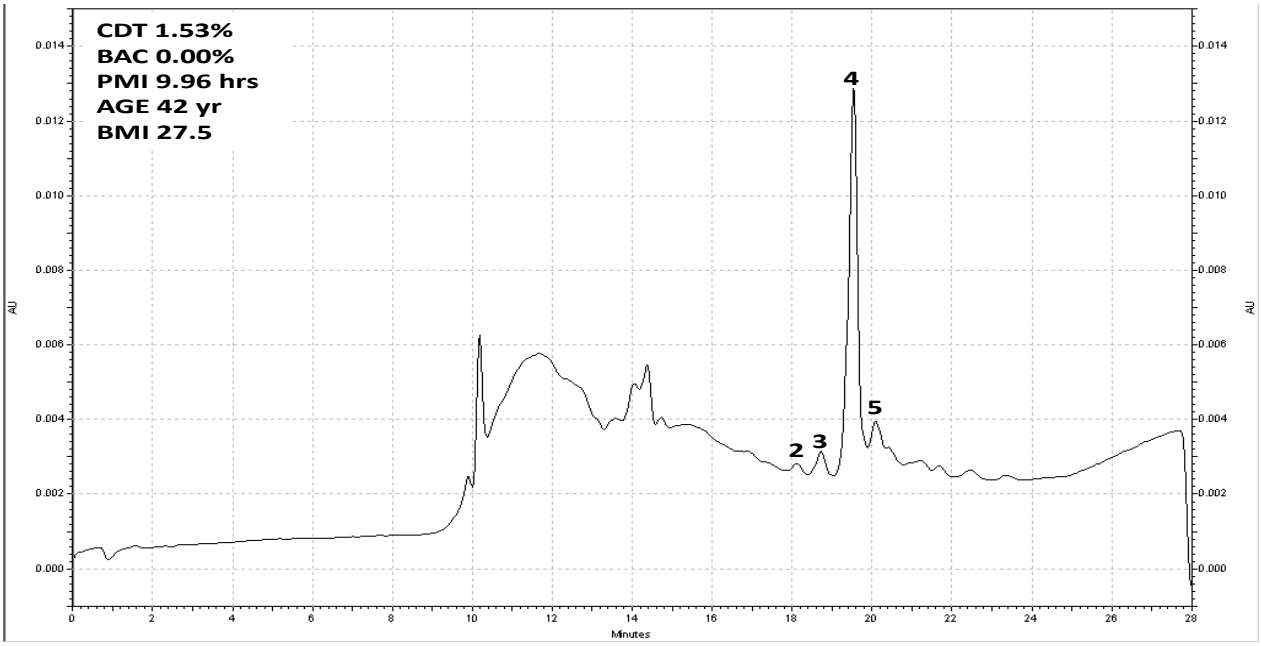

Figure 6. Electropherogram of Subject with Negative %CDT and BAC

Figure 6 shows an electropherogram of a CDT and BAC negative subject who died by suicide. There is a clear resolution of peaks with absolute migration time differences measured against the day's control to be under 1%. While this subject did not die with alcohol in their system it is not known whether they were an abuser of alcohol and it contributed to their demise. As can be the case in suicides by hanging, internal exams are not always performed (time constraints, clear cause and manner of death, family wishes) making the judgment of pathological findings consistent with chronic alcoholism impossible. The use of %CDT in this case can be critical in that it allows for the measurement of a chronic alcohol abuse marker while maintaining efficiency of the OCME.

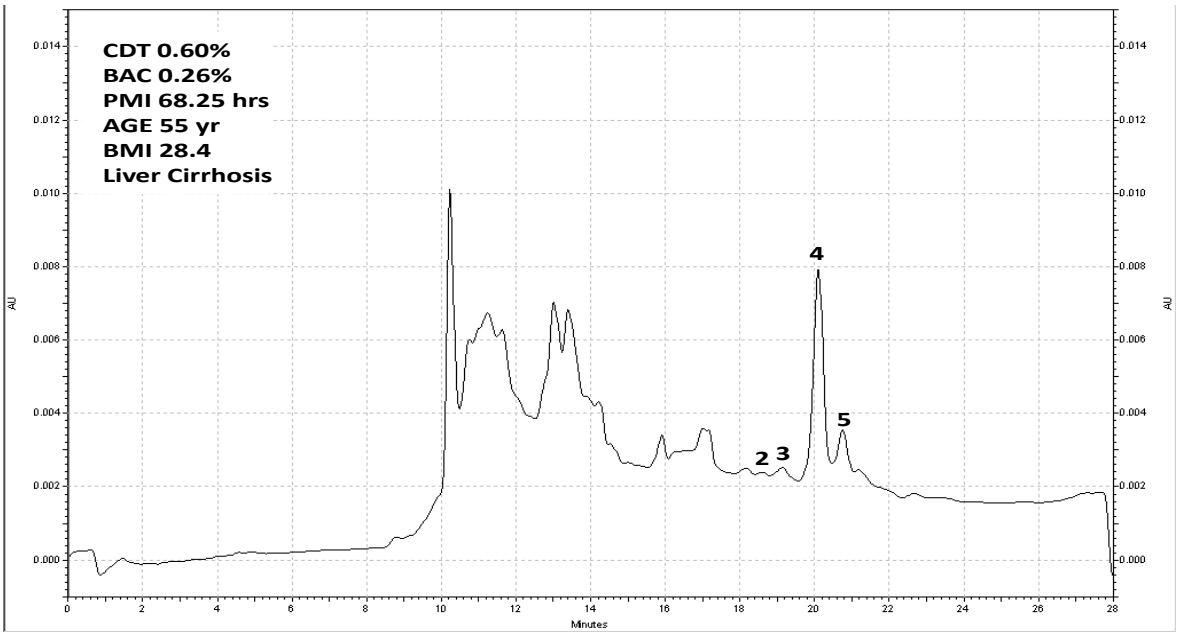

Figure 7. Electropherogram Negative for %CDT with Liver Cirrhosis

Figure 7 depicts the electropherogram from an individual who died of multiple drug toxicity and showed a negative %CDT, positive BAC. The subject suffered from cirrhosis of the liver, hypertensive cardiovascular disease, and cardiomegaly all consistent with the chronic consumption of alcohol. The interesting point is that the %CDT was low, 0.6%. While there is a peak near to the disialo-Tf glycoform integration was performed, careful not to include neighboring peaks. The relative migration times where in accordance to the daily control (<1%) lending confidence to the selected peaks. The results of this data could suggest that the person, while recently ingesting alcohol, could be abstaining by large, perhaps due to the state of their liver. While some discrepancy may be a result of poor peak resolution it is clear that the trisialo-Tf peak is higher with respect to the disialo-Tf and both peaks are not very large. The low %CDT would be consistent with these observations.

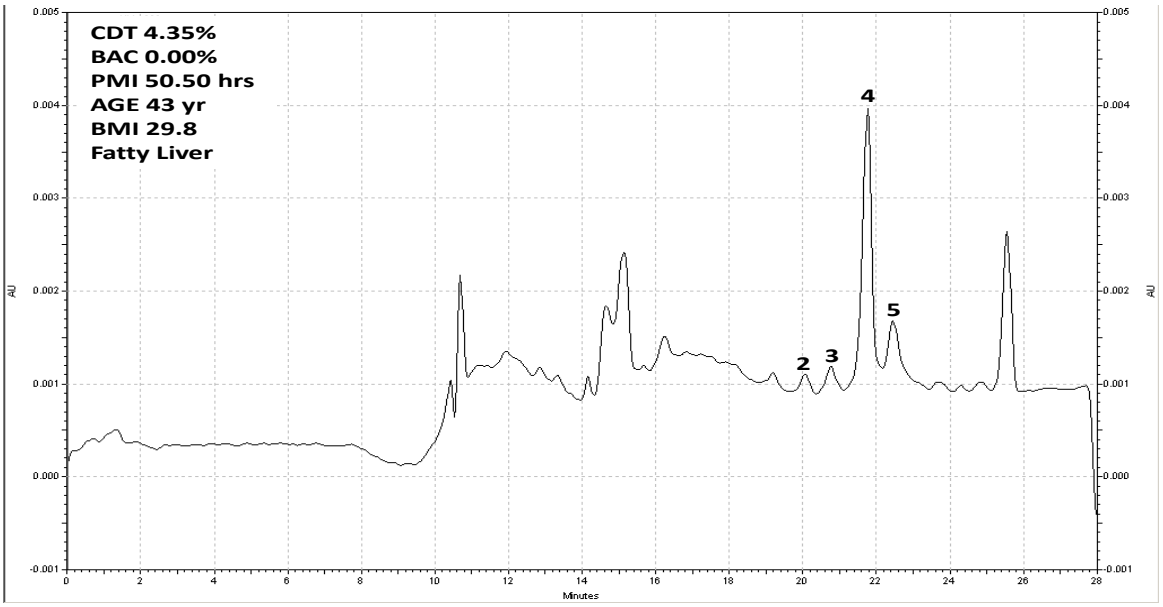

Figure 8. Electropherogram of Chronic Alcohol Abuser with Pathological Findings

Figure 8 shows an electropherogram of a subject with elevated %CDT indicative of chronic alcohol abuse ante-mortem. The subject had a history of abuse along with fatty liver at time of autopsy. While it was not a mystery that the person was an alcoholic the %CDT information supports this fact and tells the medical examiner that the subject was likely still consuming high quantities (>60g/day) up until their death.

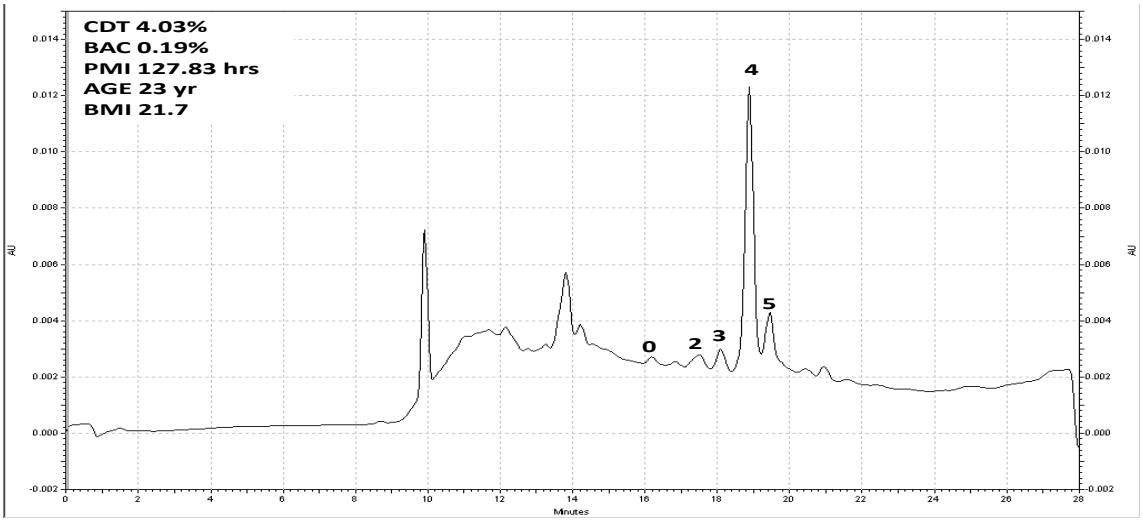

Figure 9. Electropherogram of Increased %CDT without Pathological Findings

Figure 9 shows an electropherogram of a subject with elevated levels of CDT and BAC but no pathological findings at time of autopsy. While an acute intoxication measured by BAC levels is clear, it might be of interest to know if the subject was an alcohol abuser to determine a behavioral pattern that might be helpful to investigators. The subject in the above electropherogram suffered from severe ethanol, cocaine, and heroin toxicity. %CDT allows us to infer that this was most likely not a one-of or two-of event that ended poorly but a pattern of abuse at least in reference to alcohol. Moreover the lack of pathological findings could be a result of his young age, not allowing enough time for the pathology to present and would go undiagnosed. This might be of importance, for example, if the individual was involved in other crimes or spent time around those who were.

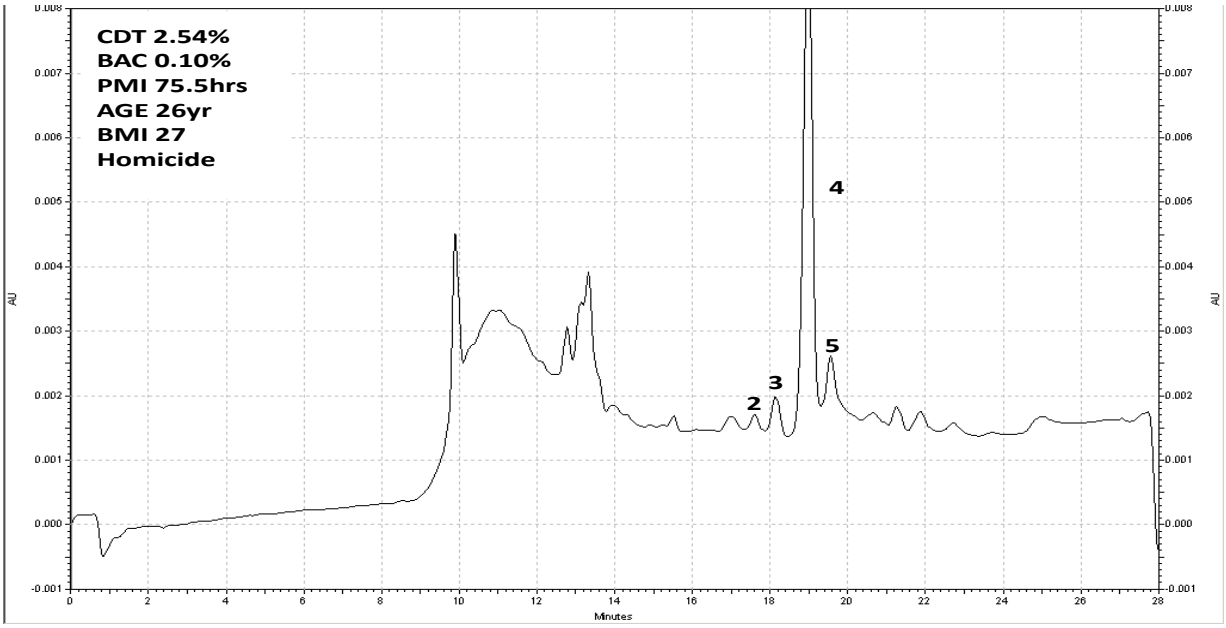

Figure 10. Electropherogram of Subject Involved In a Homicide Showing Increased BAC and CDT Levels

Another interesting case is depicted in figure 10. The subject received a stab wound of the chest and had a positive BAC at time of death as well as an elevated level of %CDT. These facts taken together can indicate a chronic abuse of alcohol and engagement in risky behaviors.

INTERPRETATION ISSUES

All samples were measured against their respective daily controls and any that did not maintain relative migration time ratios of <1% were not included. A large frustration was the myriad of interfering proteins contained in these post-mortem samples. A preliminary effort was made to find a way to precipitate out these interferences with the aim of reducing their hindrance on interpreting CDT. In brief, calcium chloride, ammonium sulfate, and ammonium persulfate were used in varying concentrations and volumes to see if incubation would clean up the sample before analysis. The pretreatment steps were often too severe (removing all proteins) and at lower levels had no real effect worth the potential of protein loss, not to mention the intense current spikes that would break capillaries even after sample dilution before injection. The reality of the method was that all proteins were precipitated in an indiscriminate and irreproducible fashion, separation was affected by current spikes even at low concentrations of salts, and quantitative analysis of %CDT would likely have been affected by this. Moving beyond salt precipitation, a major interfering protein was addressed. Hemoglobin, found in large concentrations in hemolyzed samples migrates at a similar time as the transferrin glycoforms often masking their pattern due to hemoglobin's high peaks. Hemoglobin proteins in the serum fraction of ante-mortem blood are normally contained in the red blood cells and centrifuged prior to testing, however in post-mortem samples the red blood cells are often lysed, releasing their contents. Acid treatment of hemoglobin as described in the methods section was applied to post-mortem samples prior to CDT testing to verify the effectiveness of altering the migration time of hemoglobin by resolving the tetramers into smaller species with different mobilities.

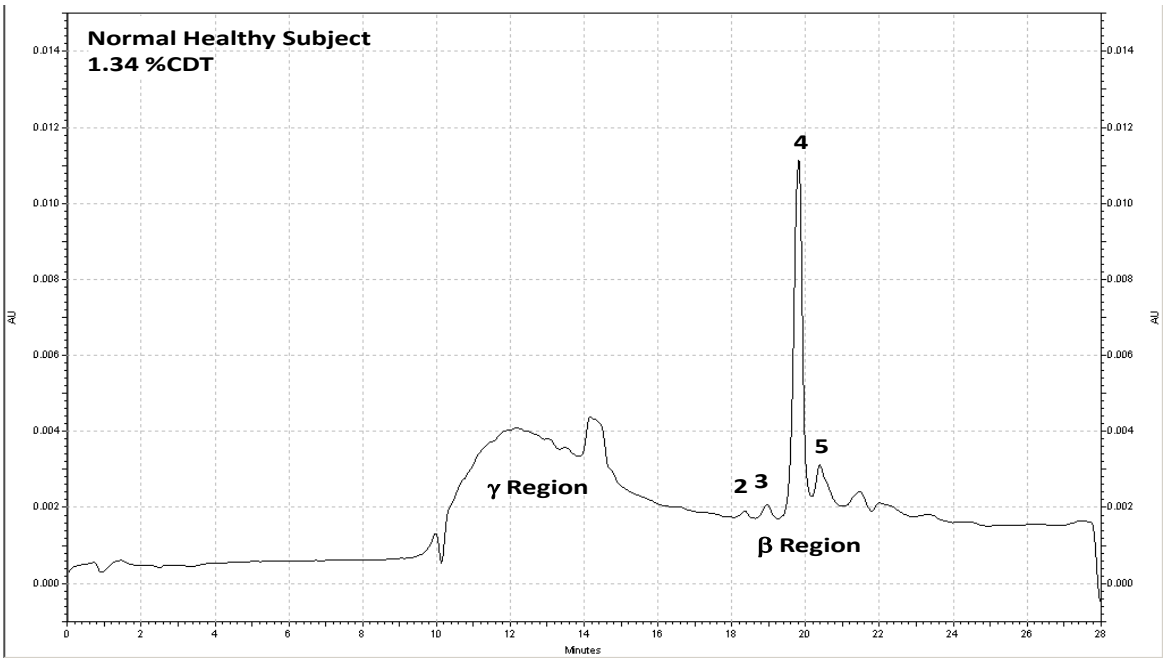

Figure 11. Electropherogram of Transferrin Glycoforms in Healthy Individual

Figure 11 depicts a normal serum distribution of CDT in a healthy subject, indicating the gamma (γ) and beta (β) regions. The gamma region of the electropherogram shows the migration of the gamma globulins found in blood. While this is a large mass of unresolved proteins it is clear that they do not interfere with the detection or measuring of CDT isoforms in the beta region. This electropherogram is generally free of other interfering proteins but in the case of post-mortem samples there are a lot that migrate at the same time as transferrin, one in particular hemoglobin (Figure 12).

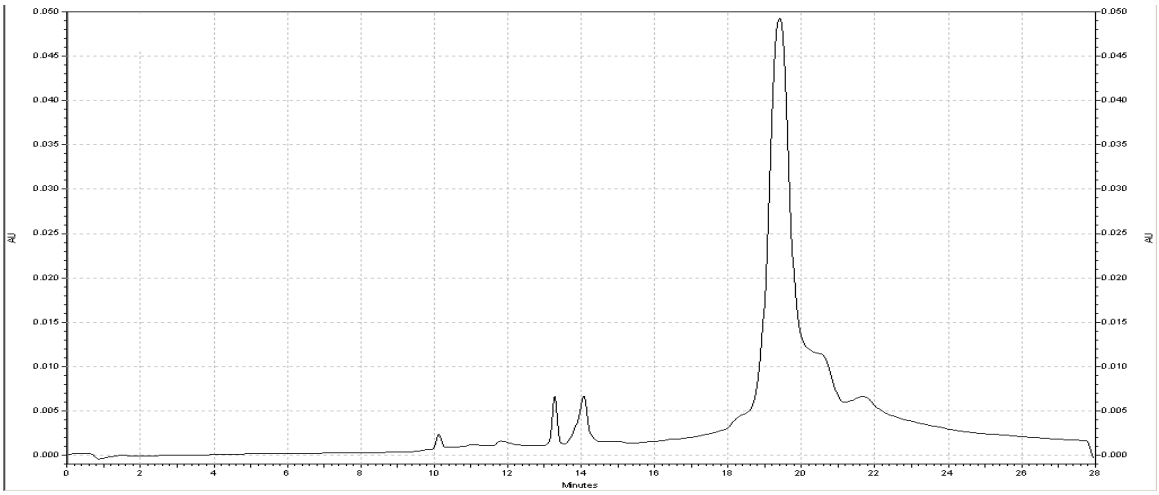

Figure 12. Electropherogram of Hemoglobin Interference in β Region

The analysis of acid treated samples was done in the same way as all other samples, the difference being the sample pretreatment discussed in the methods section and adopted from another method used previously from the lab.

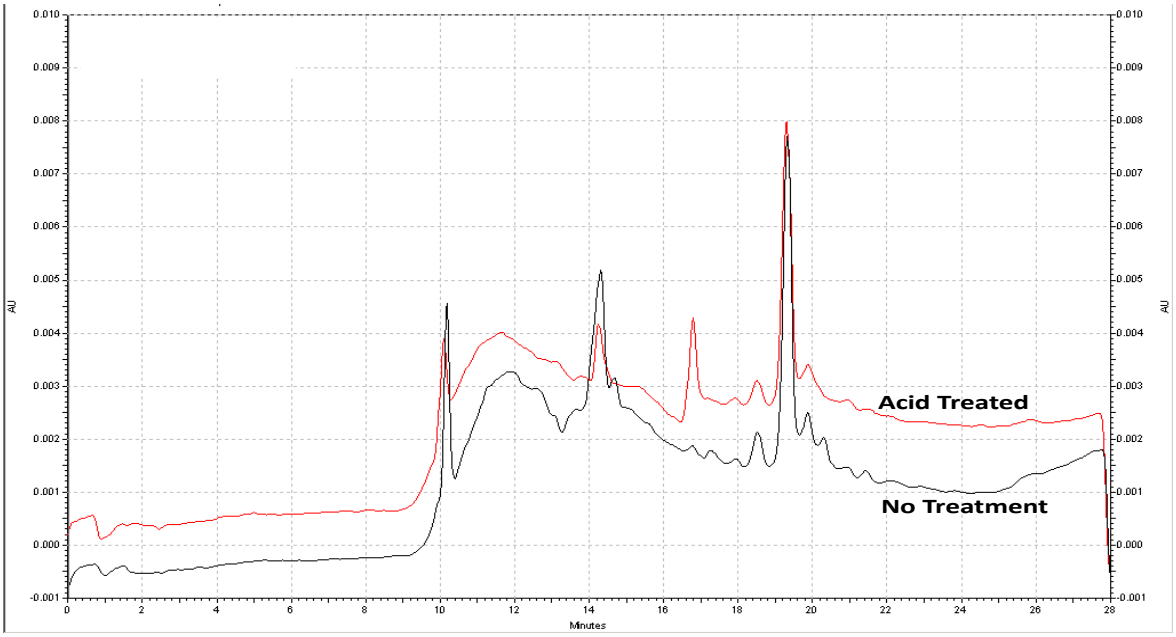

Figure 13. Electropherogram of a Sample with and Without Acid Treatment

Preliminary results were obtained for a normal control and one other sample (Figure 13) maintaining relative migration ratios <1% with daily controls. The sample in figure 13 was chosen because the pattern of

transferrin was clear, with only some low quantities of other proteins. This preliminary separation was done for two reasons, the first to make sure that while the normal serum (provided by live subject) was not affected by acid treatment neither would a post-mortem sample, and second to see if the acid treatment while developed for hemoglobin removal would be able to address any of the other proteins in the β region. The resulting electropherograms show no marked improvement in the transferrin pattern, however it at least showed no significant change in the level of %CDT measured with and without treatment implying little affect on the Tf molecules.

The lack of data obtained was in great part due to the poor separation achieved for more "ugly" samples and small number of experiments performed. In most cases a discernable transferrin pattern was not found. Conclusions based on the improvement of acid treatment to hemoglobin rich samples cannot be made as there were few cases to judge. However in general when preparing samples, those that appeared darker by eye (more hemoglobin) would often have larger precipitates after the addition of 0.1 M sodium tetraborate and ultimately lead to poorer CZE separation. More experiments adjusting parameters and choosing samples in varying degrees of hemoglobin concentrations are necessary.

CONCLUSIONS

The distinctive lack of tools available to medical examiners and investigators to report on chronic alcohol abuse at death is unfortunate. Utilizing a capillary zone electrophoresis method to separate and detect %CDT in post-mortem blood samples allows the forensic community insight into ante-mortem habits. A study of 498 samples yielded 72 subjects with interpretable electropherograms. The study revealed no significant differences between races, genders, PMI, or BMI in general for an increase of %CDT. A significant correlation could be found in subjects with anatomo-pathological signs of chronic alcohol abuse and increased %CDT as well as a higher frequency of persons involved in accidental deaths. This fact corroborates with the thought that alcoholics engage in riskier behaviors than normal persons.

While not a large percentage of the total samples, the value of the information gained can be immeasurable. When an autopsy is not

performed, CDT can still be measured by taking a small sample of blood and could be helpful in determining cause and manner of death. In addition, further investigation into sample pretreatment options and decreasing PMI by having on site centrifugation or short travel times will only improve the rate of interpretable data. Capillary zone electrophoresis is a useful tool to determine %CDT in post-mortem samples.

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